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TRANSGENIC PLANT WITH ELEVATED CONTENT OF SECONDARY SUBSTANCES

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TRANSGENIC PLANTS WITH ELEVATED CONTENT OF SECONDARY SUBSTANCES

[Transgene Pflanzen mit erhöhtem Gehalt an Sekundärstoffen]

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Documents taken into consideration for the evaluation of the patentability:

DE 42 34 131 A1
DE 41 30 986 A1
DE 41 17 747 A1
WO 93 15 599 A1
M. J. C. Rhodes: Physiological voles for secondary metabolites in plants: some progress, many outstanding problems, plant Molecular Biology 24, 1-20, 1994;

R. Chasan: Phytochemical Forecasting, The Plant Cell 6, 3-9, 1994

The present invention relates to a transgenic plant with at least one heterologous DNA sequence having the characteristics of Claim 1. The plant should have an elevated content of certain secondary substances. Moreover, the invention relates to a method for its production, as well as to its use in forest, pasture, lawn, decorative plant or useful plant cultivation.

Vegetal secondary substances play an important role in the plant's defense against pathogens, feeding pests or parasites (see, for example, B. Rhodes, Plant Molecular Biology 24 (1994), 1-20; Chasan, Plant Cell 6 (1994), 3-9).

The German Patent [Offenlegungsschrift] DE-OS 41 17 747 describes transgenic plants, which contain new caffeoyl-CoA 3-O-methyltransferase gene isolated from plants. The expression product caffeoyl-CoA 3-O-methyltranserase of this gene catalyzes the methylation of caffeoyl-CoA in a biosynthetic path which leads from trans-4-cumaroyl-CoA to trans-feruloyl-CoA. The pathogen resistance effect here is not based on the production of potentially toxic metabolites in the transgenic plants, rather the goal is to synthesize predominantly insoluble compounds that have no antibiotic effect, and that are intended to function as physical barriers or to prevent a pathogen-induced, enzymatic lysis of cell wall polysaccharides by acylation. The Patent [Offenlegungsschrift] DE-OS 41 30 986 describes transgenic plants containing new genes for pinosylvin synthase isolated from plants. In particular, one can consider using as plants from which the pinosylvin synthase gene can be isolated any monocotyledonous or dicotyledonous plants. The Patent [Offenlegungsschrift] WO 93/15 599 A1 describes transgenic plants which contain phenyloxidase genes from angiosperms ("covered by seed"). The polyphenoloxidases which occur in angiosperms catalyze the oxidation of phenols to quinones when there is an excess of O_2. The Patent [Offenlegungsschrift] DE-OS 42 34 131 describes transgenic plants which contain two different genes, where the products of the genes are the enzymes "quitinase" and "glucanase," the protein "protein synthesis inhibitor" or the polypeptide "antifungal protein." These gene products themselves are responsible for the pathogen resistance effect and do not produce any secondary substances.

However, no methods have become known to date for clearly increasing the content of secondary substances over a longer time period and consequently achieving an increased pathogen resistance and/or an increased resistance against feeding pests or parasites. Today, plant protection is based primarily on the treatment of plants with different pesticides, the toxicology and ecological effects of which are problematic in many cases.

Thus, the invention is based on the problem of preparing a plant which per se is resistant to pathogens and/or to feeding pests or parasites, for the purpose of at least partially limiting the use of pesticides and thus improving the plant protection from the toxicological and ecological points of view.

This problem is solved according to the invention by producing a transgenic plant that has at least one heterologous DNA sequence, which codes for at least one polypeptide with enzymatic activity, in which the polypeptide is expressed, present in an enzymatically active form and produces at least one secondary substance dissolved enzymatically in an effective antiviral and/or bactericidal and/or fungicidal and/or insecticidal and/or repellent concentration, where the heterologous DNA sequence originates from bacteria.

The expression "transgenic plant" or "plant" refers to the entire plant as such, as well as to its parts, such as the root, stem, leaf, organ-specific tissues or cells, its reproductive material, particularly seeds, and its seedlings.

The expression also covers the Gymnosperinae [sic; Gymnospermae], the Monocotyledonae and Dicotyledonae, particularly useful plants, such as cereals, for example, barley, corn, wheat, corn [sic], barley, rice, oats and millet; starchy tubers and roots, for example, potatoes, yams and cassava; sugar plants, for example, sugar cane and sugar beet; pod fruits, for example, beans, peas and chickpeas; oily and fatty fruits, for example, soybeans, peanuts, sunflower seeds, oil tree, rapeseeds and coconuts; vegetables, for example, tomato, cabbage, onions, cucumber, carrots and salad; fruits, for example, grapes, citrus fruits, banana, apple, pear, peach and pineapple; nut-like fruits, for example, walnuts, hazelnuts, almonds and cashew nuts; semiluxury food plants, for example, tobacco, coffee, tea, cacao; species that produce plant fibers, for example, cotton, jute and flax; species used in forestry, for example, firs, oaks and poplars; and plant species for the production of raw materials, for example, *Hevea brasiliensis* (rubber) and jojoba.

The term "heterologous DNA sequence" denotes a DNA sequence that originates from a source of prokaryotic origin, for example, *Escherichia coli*, including archaebacteria. The coding region of the heterologous DNA sequence can contain coding ("exons") and noncoding ("introns") sections. Furthermore, the heterologous DNA sequence can contain regulatory sections such as promoters, enhancers and termination sequences. It is preferred to use a heterologous DNA sequence that does not contain introns.

In addition, this term covers chimeric DNA sequences, as well as synthetic or semisynthetic DNA sequences.

In a preferred embodiment, the DNA sequence contains the ubiC gene from *Escherichia coli*; see Figure 1.

The terms "polypeptide with enzymatic activity" denote a polypeptide which is coded from the above-defined heterologous DNA sequence, where the coding sequence can be degenerate in accordance with the genetic code.

The primary transcript can be translated directly or it can be converted into a translatable mRNA by "splicing." Furthermore, the primary translation product can undergo a post-translation modification for the formation of the enzymatically active polypeptide, for example, by cleaving off a signal sequence, by enzymatic cleavage of the inactive "precursor" for conversion into the enzymatically active form or by modification of the side chains of the polypeptide, for example, by phosphorylation or glycosylation.

Preferred examples of these polypeptides are the enzyme chorismate pyruvate-lyase from *Escherichia coli*, whose primary sequence is represented in Figure 2, and the enzyme isochorismate pyruvate-lyase (= salicylate synthase).

According to the invention, these polypeptides, using substrates present in the transgenic plant, produce enzymatic secondary substances which preferably also occur in the wild type in small quantities ("plant endogenous secondary substances"). Unexpectedly, in the transgenic plant according to the invention the secondary substances become enriched to a biologically active concentration, where the enrichment is the result of anabolic or catabolic secondary reactions or of deposition in certain cell compartments.

The concentration of the secondary substances that become enriched in the transgenic plant according to the invention exhibits a clear antiviral and/or bactericidal and/or fungicidal and/or insecticidal and/or repellent effect. It is preferred for this biologically effective concentration of the secondary substances (unit: µg secondary substance/g fresh weight of the plant) to be at least 10 times, preferably at least 50 times, and most advantageously at least 100 times, the concentration of these substances in the wild type plants.

The term "secondary substance" denotes substances contained in plants, which are part of the secondary metabolism, as well as their derivatives, which are produced either by follow-up reactions in the plant cell, for example, hydroxylation, methylation, glycosylation, etherification, esterification or polymerization, or which are produced directly enzymatically from the above-defined polypeptide. Thus, either the secondary substance (or its derivative), which is produced according to the invention, per se or [a] derivative(s) produced by subsequent reactions produce the above-defined biological effect.

Examples of secondary substances are alkaloids, isoprenoids, phenol derivatives, phenylpropanes, quinones, cumarines, lignin and flavonoids. It is preferred that the secondary substances be phenol derivatives, particularly p-hydroxybenzoic acid and its derivatives.

An additional objective of the present invention relates to a method for the production of the transgenic plants according to the invention, where a plant cell is transformed by stable

integration of the heterologous DNA sequence into the genetic material, and the transformed plant cell is regenerated into the transgenic plant.

Methods for the production of transgenic plants are known in the state of the art. For example, the Ti plasmid or a binary plasmid system in *Agrobacterium tumefaciens* can be used as a vector for the stable integration of the heterologous DNA sequence in the genetic material of the transgenic plant according to the invention. Moreover, the heterologous DNA sequence can also be introduced, for example, via the Ri plasmid of *Agrobacterium rhizogenes*, by direct gene transfer with polyethylene glycol, by electroporation or by particle bombardment into the genetic material of the transgenic plant.

Another objective of the present invention relates to the use of the transgenic plant according to the invention as a plant that is resistant to pathogens and/or to feeding pests and/or parasites in forest, pasture, lawn, decorative plant and useful plant cultivation.

Examples of pathogens are, among the viruses, the tobacco mosaic virus and the cabbage mosaic virus; among the bacteria, *Erwinia amylovora*, *Pseudomonas syringae*, *Corynebacterium michiganense* and *Xanthomonas campestris*; and, among the fungi, *Phytophtora infestans*, *Claviceps purpurea*, *Botrytis cinerea* and *Ustilago maydis*. Among the feeding pests and parasites, the following are mentioned particularly: nematodes, aphids, beetles and butterfly caterpillars.

Furthermore, the present invention relates to the use of the above-defined heterologous DNA sequence for the production of transgenic plants according to the invention.

In the drawing:

Figure 1 shows the coding 495 bp long nucleic acid sequence of the ubiC gene from *Escherichia coli*.

Figure 2 shows the 165 aa long amino acid sequence of chorismate pyruvate-lyase from *Escherichia coli*.

Figure 3 is a schematic representation of the construction of the transformation vectors pROK-ubiC and pROK-TP0-ubiC with the ubiC gene from *Escherichia coli*.

The following example explains the invention:

The phenol derivative p-hydroxybenzoic acid (PHB) is a key metabolite, both in bacteria and in higher plants, in the biosynthesis of ubiquinone, an electron-transferring compound in the respiration chain (Pennock and Threlfall (1983), in: Biosynthesis of Isoprenoid Compounds (Porter and Spurgeon, Editors), Vol. 2, 191-203). In both groups of organisms, PHB is produced from chorismate, but by completely different pathways. In plants, chorismate is converted first during the aromatic amino acid biosynthesis via prephenate into phenylalanine and then it is converted via cinnamic acid and p-cumaric acid to PHB (Heide et al., Phytochemistry 28 (1989), 2643-2645). In bacteria, on the other hand, chorismate is metabolized in a single step by the

enzyme chorismate pyruvate-lyase to PHB and pyruvate. The gene ubiC that codes for this enzyme has been cloned from *Escherichia coli* (Siebert et al., FEBS Letters 307 (1992), 347-350); see Figures 1 and 2.

1) Construction of the transformation vector with the ubiC gene from *Escherichia coli*

For the construction of a transformation vector, the ubiC gene contained in pUBIC (Siebert et al., Microbiology 140 (1994), 897-904) is cleaved with EcoRI and SalI, the ends are filled with Klenow enzyme, followed by cloning in the binary-plant expression vector pROK1 (Bevan et al., EMBO J. 4 (1985), 1921-1926), which was linearized with BamHI beforehand and the ends of which are also filled with Klenow enzyme. This vector allows the selection of the transgenic plants by means of a kanamycin resistance gene, where the ubiC gene is under the control of a 35S-CaMV promoter. The transformation vector produced in this manner is called pROK-ubiC; see Figure 3.

A modified ubiC gene is produced in such a manner that, while preserving the reading frame at the 5' end with a plasmidic transit peptide (Sugita et al., Mol. Gen. Genet. 209 (1987), 247-256) that originates from the small subunit of the ribulose-bisphosphate carboxylase, it is fused via the KpnI cleavage site, resulting in the construct pTP0-ubiC. The pTP0-ubiC containing fusion gene TPubiC is then cloned as described above into the vector pROK1 to obtain the transformation vector pROK-TP0-ubiC; see Figure 3.

2) Transformation of tobacco plants

For the transformation of tobacco plants (cultivar Petite Havanna SR1), leaves of tobacco cultivated under sterile conditions are cut into approximately 0.5 x 0.5 cm pieces and immersed for at least one minute in a culture of *Agrobacterium tumefaciens* (LBA4404 in each case with the transformation vectors described under point (1)), where the culture was briefly centrifuged beforehand and resuspended in identical volumes of sterile tap water. The leaf pieces are plated with their bottom sides facing up on SHI medium (in one liter 4.6 g salts according to Murashige and Skoog (Physiol. Plant. 15 (1962), 473-497), 30 g sucrose, 1 mg 6-benzylaminopurine, 0.1 mg naphthylacetic acid, 100 mg inositol, 10 mg thiamine, 1 mg pyridoxine, 1 mg nicotinic acid and 7 g agarose) in Petri dishes, and cocultured at 25°C for three days with the agrobacteria. Care should be taken here that the quantity of light is not excessively large (scattered light, approximately 1000-3000 lux).

The selection against agrobacteria and untransformed leaf parts is carried out by transferring the pieces of leaves onto $SHI_{Cef250,Kan100}$ medium (composition like SHI medium with the addition of 250 mg/L cefotaxim and 100 mg/L kanamycin).

After 4-6 weeks, the regenerated buds, which have formed at the cutting sites, are separated and inserted in $_{Cef250,Kan100}$ medium (composition like the corresponding SHI medium, but without the addition of naphthylacetic acid, 6-benzylaminopurine, inositol, thiamine, pyridoxine and nicotinic acid) in 450 mL canning jars for rooting. When the plants have reached a height of approximately 10 cm, they are carefully taken out by the roots from the agar medium and placed into sterilized soil.

3) Analysis of the transgenic tobacco plants to determine their PHB content

Leaf material of the transgenic plants produced under point (2) is ground under liquid nitrogen in a mortar. For the extraction of the PHB in free form in the plants, the powdered material is suspended in 0.75M sodium acetate solution at pH 4.0 and extracted with ethyl acetate. The organic phase is removed, followed by evaporation to dryness, dissolution in methanol/water/formic acid (30:69.3:0.7), and examination by HPLC chromatography.

For the determination of the bound PHB (for example, esters, glucosides), the powdered plant material is hydrolyzed in 1M HCl for one hour at 80°C, extracted with ethyl acetate, and analyzed as described above.

In comparison to wild type plants, the transformed plants present a content of free PHB which is increased by a factor of 50 (2.3 µg/g fresh weight) and a content of bound PHB which is increased by a factor of 1150. In the transgenic plants, approximately 50% of the PHB is bound to glucosides. The PHB glucoside content of the plant is approximately 0.3 mg/g fresh weight. In addition, other phenolic substances also become enriched.

4) Biological effect of the elevated concentration of certain secondary substances in the transgenic tobacco plants of the tobacco-mosaic virus (TMV)

For the TMV resistance test, a suspension of the viruses is applied with diatomaceous earth under a slight pressure with a brush onto the leaves of transgenic and wild tobacco plants (cultivar Petite Havanna SR1).

Because the virus spreads systemically in Petite Havanna plants without forming any local lesions, the leaves of the tobacco plants are ground in a mortar after approximately 10 days, and the extract is applied as described above on the leaves of the tobacco cultivar *Nicotiana tabacum* cv. Xanthi. After approximately three days, the local lesions which occur in these plants are observed.

As a result, it has been shown that (i) in the untransformed control plants of *N. tabacum* cv. Petite Havanna, a high TMV titer can be established, (ii) the TMV multiplication in transgenic plants that have been transformed with pROK-TP0-ubiC is decreased clearly, and (iii)

in transgenic plants that have been transformed with pROK-ubiC essentially no TMV can be detected.

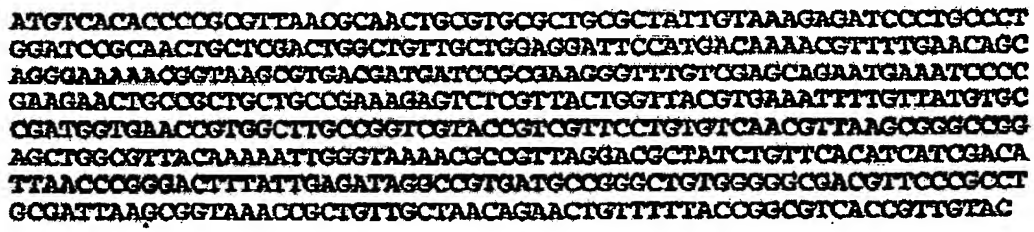
In summary, one can observe that the transgenic tobacco plants according to the invention, which contain the ubiC gene from *Escherichia coli*, present a clearly elevated pathogen resistance compared to the wild type plants and that they do not form, in the plant, new substances with unknown effect and toxicology, rather they unexpectedly contain an elevated quantity of p-hydroxybenzoic acid (a substance with known properties, which occurs naturally in all organisms) and its derivatives, particularly p-hydroxybenzoic acid glucoside, which are responsible for resistance to pathogen, or play an important role.

Claims

1. Transgenic plant having at least one heterologous DNA sequence, which codes for at least one polypeptide with enzymatic activity, in which the polypeptide is expressed, is present in an enzymatically active form, and enzymatically produces at least one secondary substance at an effective antiviral and/or bactericidal and/or fungicidal and/or insecticidal and/or repellent concentration, where the heterologous DNA sequence originates from bacteria.

2. Transgenic plant according to Claim 1, where the heterologous DNA sequence originates from *Escherichia coli*.

3. Transgenic plant according to Claim 1 or 2, where the heterologous DNA sequence has the following sequence:



4. Transgenic plant according to one of Claims 1-3, where the polypeptide is the enzyme chorismate pyruvate-lyase or the enzyme isochorismate pyruvate-lyase (= salicylate synthase).

5. Transgenic plant according to one of Claims 1-4, where the secondary substance is a plant endogenous substance.

6. Transgenic plant according to one of Claims 1-5, where the secondary substance is a phenol derivative.

7. Transgenic plant according to one of Claims 1-6, where the secondary substance is p-hydroxybenzoic acid and/or its derivative(s).

8. Transgenic plant according to one of Claims 1-7, where the concentration of the secondary substance is at least 10 times the concentration of the secondary substance in the wild type.

9. Method for the production of a transgenic plant according to one of Claims 1-8, in which a plant cell is transformed by the stable integration of the heterologous DNA sequence into the genetic material, and the transformed plant cell is regenerated into the transgenic plant.

10. Use of the transgenic plant according to one of Claims 1-8 as a plant which is resistant to pathogens and/or to feeding pests and/or parasites in forest, pasture, lawn, decorative plant or economic plant cultivation.

11. Use of a heterologous DNA sequence, which codes for at least one polypeptide with enzymatic activity, for the production of a transgenic plant having at least one plant endogenous secondary substance produced by the polypeptide at an effective antiviral and/or bactericidal and/or fungicidal and/or insecticidal and/or repellent concentration.

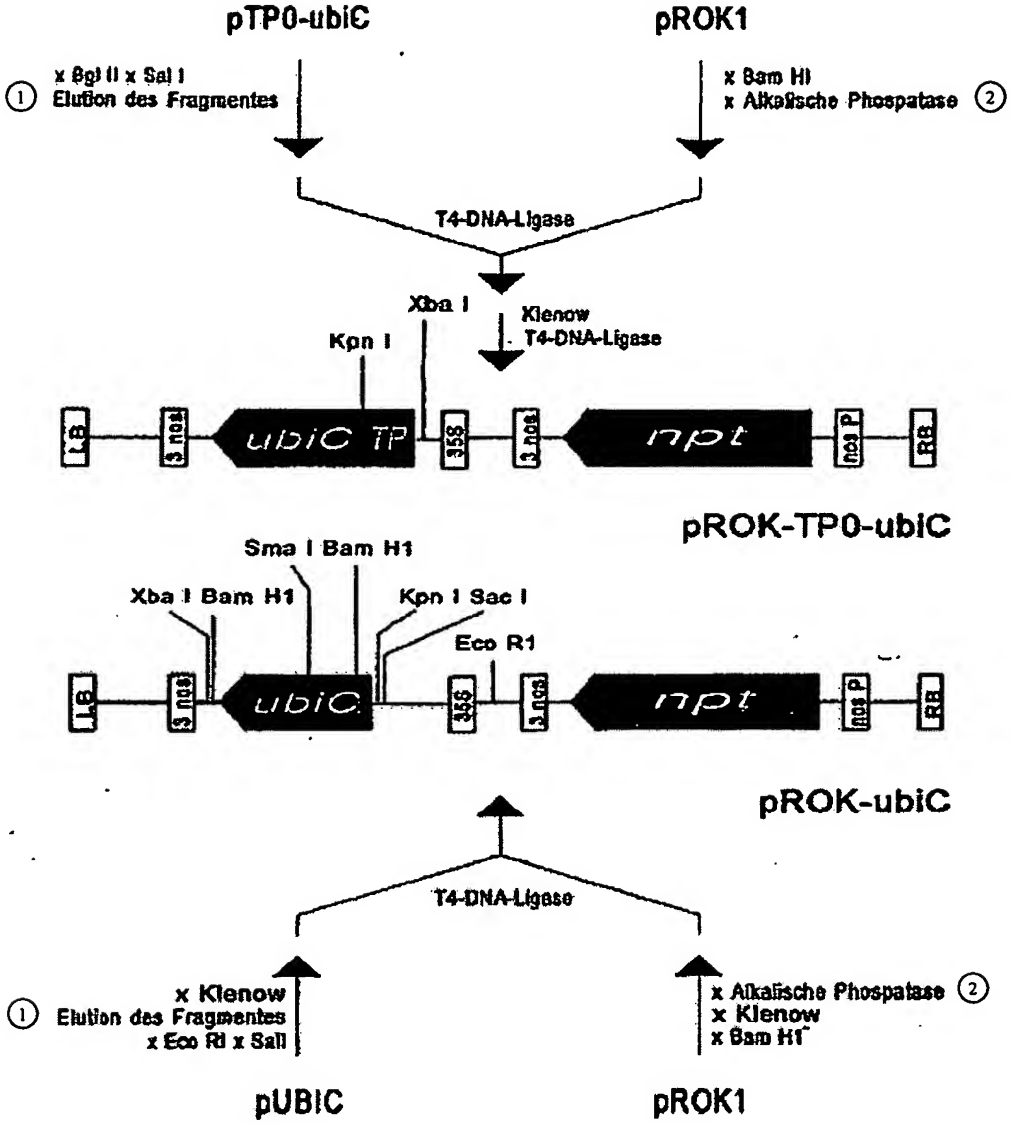
Fig. 1

ATGTCACACCCCGCGTTAACGCAACTGCGTGCGCTGCGCTATTGTAAAGAGATCCCTGCCCT
GGATCCGCAACTGCTCGACTGGCTGTTGCTGGAGGATTCCATGACAAAACGTTTTGAACAGC
AGGGAAAAACGGTAAGCGTGACGATGATCCGCGAAGGGTTTGTCGAGCAGAATGAAATCCCC
GAAGAACTGCCGCTGCTGCCGAAAGAGTCTCGTTACTGGTTACGTGAAATTTTGTTATGTGC
CGATGGTGAACCGTGGCTTGCCGGTCGTACCGTCGTTCCTGTGTCAACGTTAAGCGGGCCGG
AGCTGGCGTTACAAAAATTGGGTAAAACGCCGTTAGGACGCTATCTGTTCACATCATCGACA
TTAACCCGGGACTTTATTGAGATAGGCCGTGATGCCGGGCTGTGGGGGCGACGTTCCCGCCT
GCGATTAAGCGGTAAACCGCTGTTGCTAACAGAACTGTTTTTACCGGCGTCACCGTTGTAC

Fig. 2

M	S	H	P	A	L	T	Q	L	R	A	L	R	Y	C	K	E	I	P	A	L
D	P	Q	L	L	D	W	L	L	L	E	D	S	M	T	K	R	F	E	Q	Q
G	K	T	V	S	V	T	M	I	R	E	G	F	V	E	Q	N	E	I	P	E
E	L	P	L	L	P	K	E	S	R	Y	W	L	R	E	I	L	L	C	A	D
G	E	P	W	L	A	G	R	T	V	V	P	V	S	T	L	S	G	P	E	L
A	L	Q	K	L	G	K	T	P	L	G	R	Y	L	F	T	S	S	T	L	T
R	D	F	I	E	I	G	R	D	A	G	L	W	G	R	R	S	R	L	R	L
S	G	K	P	L	L	L	T	E	L	F	L	P	A	S	P	L	Y			

Fig. 3



Key: 1 Elution of the fragment
2 x Alkaline phosphatase